SUN1 and SUN2 play critical but partially redundant roles in anchoring nuclei in skeletal muscle cells in mice

Kai Lei[a,1], Xiaochang Zhang[a,1], Xu Ding[a], Xue Guo[a], Muyun Chen[a], Binggen Zhu[a,2], Tian Xu[a,b], Yuan Zhuang[a,c], Rener Xu[a,3], and Min Han[a,d,3]

[a]Institute of Developmental Biology and Molecular Medicine, School of Life Science, Fudan University, Shanghai 200433, China; [b]Howard Hughes Medical Institute and Department of Genetics, Yale University School of Medicine, New Haven, CT 06520; [c]Department of Immunology, Duke University Medical Center, Durham, NC 27710; and [d]Howard Hughes Medical Institute and Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, CO 80309

Edited by Eric N. Olson, University of Texas Southwestern Medical Center, Dallas, TX, and approved May 1, 2009 (received for review November 28, 2008)

How the nuclei in mammalian skeletal muscle fibers properly position themselves relative to the cell body is an interesting and important cell biology question. In the syncytial skeletal muscle cells, more than 100 nuclei are evenly distributed at the periphery of each cell, with 3–8 nuclei anchored beneath the neuromuscular junction (NMJ). Our previous studies revealed that the KASH domain–containing Syne-1/Nesprin-1 protein plays an essential role in anchoring both synaptic and nonsynaptic myonuclei in mice. SUN domain–containing proteins (SUN proteins) have been shown to interact with KASH domain–containing proteins (KASH proteins) at the nuclear envelope (NE), but their roles in nuclear positioning in mice are unknown. Here we show that the synaptic nuclear anchorage is partially perturbed in *Sun1*, but not in *Sun2*, knockout mice. Disruption of 3 or all 4 *Sun1/2* wild-type alleles revealed a gene dosage effect on synaptic nuclear anchorage. The organization of nonsynaptic nuclei is disrupted in *Sun1/2* double-knockout (DKO) mice as well. We further show that the localization of Syne-1 to the NE of muscle cells is disrupted in *Sun1/2* DKO mice. These results clearly indicate that SUN1 and SUN2 function critically in skeletal muscle cells for Syne-1 localization at the NE, which is essential for proper myonuclear positioning.

KASH domain | Nesprin | neuromuscular junction | nuclear envelope protein | Syne-1

Proper nuclear positioning relative to the cell body is important for many cellular processes during animal development. Mammalian skeletal muscle fibers are giant syncytial cells, each containing more than 100 nuclei. Each muscle fiber contains a neuromuscular junction (NMJ) that starts to form at approximately embryonic day 14 (E14) in mice (1). Following a multistep process involving the function of the agrin-MuSK-rapsyn-AChR pathway, the motor nerve terminal and the postsynaptic membrane become highly differentiated, ensuring reliable terminal transmission. The nuclei in the muscle fibers are positioned in a nonrandom manner, with 3–8 nuclei (synaptic nuclei) clustered beneath each NMJ and other nuclei (nonsynaptic nuclei) distributed evenly along the cell membrane (1, 2). How synaptic and nonsynaptic nuclei properly position themselves within each muscle fiber and the consequences of disrupting the nuclear positioning are attractive problems to investigate. Recent studies have indicated that KASH (Klarsicht/ANC-1/Syne homologue) domain–containing and SUN domain–containing proteins (KASH/SUN proteins) form a complex at the nuclear envelope (NE) for various cellular functions (3–6). Whereas a mammalian KASH protein has been found to have a critical function in myonuclear anchorage (7–9), the roles of SUN proteins in this process remain unclear.

The SUN domain was first defined as a domain of shared homology between Sad1 in *Schizosaccharomyces pombe* and UNC-84 in *Caenorhabditis elegans* (10). SUN proteins are conserved inner nuclear membrane proteins containing at least 1 transmembrane domain and a C-terminal SUN domain that is localized inside the lumen of the NE (4, 11). The functions of SUN proteins in nuclear positioning were first revealed by genetic analysis of the *unc-84* gene in *C. elegans*. In *C. elegans*, UNC-84 has been shown to recruit KASH proteins ANC-1 and UNC-83 to the outer NE for critical functions in both nuclear anchorage and nuclear migration (10, 12, 13). The conserved KASH domain of UNC-83 physically interacts with the SUN domain of UNC-84 in the lumen of NE (14). Similarly, in flies, the SUN protein Klaroid is required for the localization of KASH protein Klarsicht to the NE and for nuclear migration in the eye (15). Genetic studies in the worm and yeast also have indicated roles of SUN proteins in mediating the interactions between the NE and centrosome, centromeres, and telomeres at various stages of mitotic and meiotic cells (16–22).

Mammalian SUN1 and SUN2 proteins were first identified as homologues of *C. elegans* UNC-84 (10). SUN1 and SUN2 are broadly distributed in mouse tissues, whereas 2 other, more recently identified SUN proteins, SUN3 and SPAG4, appear to be expressed in a limited amount of tissues (4, 23–25). In tissue culture cells, SUN1 and SUN2 have been shown to be inner NE proteins with their N-terminal domains localized in the nucleoplasm and the C-terminal SUN domains in the lumen of the NE (24, 26–28). We have previously shown that SUN1 mediates the telomere–NE interactions during meiosis; loss of *Sun1* function disrupts the telomere–NE attachment, homologue pairing, and synapsis formation, leading to the abolishment of gametogenesis (29). Analysis of a recently generated $Sun1^{-/-}$ mouse strain has linked the SUN1 function to piRNA expression in germ cells (30).

The KASH domain is a conserved protein motif of about 60 residues located at the C terminus of KASH proteins (13). Mammalian Syne-1/Nesprin-1 and Syne-2/Nesprin-2 belong to a family of giant KASH proteins (>6,800 residues) that includes ANC-1 in *C. elegans* and MSP-300 in *Drosophila*, each of which also contains actin-binding domains at the N terminus and a

Author contributions: K.L., X.Z., R.X., and M.H. designed research; K.L., X.Z., X.D., X.G., B.Z., and R.X. performed research; K.L., X.Z., X.D., M.C., T.X., and Y.Z. contributed new reagents/analytic tools; K.L., X.Z., and X.G. analyzed data; and K.L., X.Z., R.X., and M.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]K.L. and X.Z. contributed equally to this work.

[2]Present address: Department of Physiology and Psychiatry, Tongji University School of Medicine, Shanghai 200092, China.

[3]To whom correspondence should be addressed. E-mail: rener_xu@fudan.edu.cn or mhan@colorado.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0812037106/DCSupplemental.

large central region with mostly spectrin-like repeats (3, 5). Consistent with the studies in *C. elegans*, SUN1 and SUN2 proteins have been shown to be necessary for the localization of the mammalian KASH protein Syne-2/Nesprin-2 to the NE in tissue culture cells (24, 27). A role for Syne-1/Nesprin-1 in nuclear positioning in skeletal muscle cells was revealed in our previous genetic analysis (7, 8). Overexpression of the KASH domain–containing C-terminal fragment of either Syne-1 or Syne-2 partially damages the anchorage of synaptic nuclei; however, deletion of the KASH domain of Syne-1, but not of Syne-2, severely disrupts both synaptic and nonsynaptic nuclear anchorage. A deletion of the N-terminal actin binding domain of Syne-2/Nesprin-2 also has been generated in mice and shown to cause defects in NE architecture and skin development (31).

In this study, we explored the roles of SUN proteins in myonuclear anchorage in mice carrying single or double mutations in the *Sun1* and *Sun2* genes. Our results indicate that the 2 SUN proteins play critical roles in recruiting Syne-1/Nesprin-1 to the NE for both synaptic and nonsynaptic nuclear anchorage. These results provide important insights into the mechanism underlying the functions of the KASH–SUN complexes in animal development.

Results

The Anchorage of Synaptic Nuclei Is Partially Perturbed in *Sun1* Knockout Mice. The SUN1 protein is highly expressed and localized on the NE in skeletal muscle cells (29). To elucidate the potential function of SUN1 in myonuclear positioning, we examined whether a *Sun1* deletion would disrupt the localization of Syne-1 onto the NE, as well as myonuclear positioning. Syne-1 has been shown to localize at a significantly higher level at the NE of synaptic nuclei than the NE of nonsynaptic nuclei (32). A major component of the NMJ is the acetylcholine receptor (AChR), which can be specifically bound by a chemical compound called alpha-bungrotoxin (α-BTX). NMJ is commonly visualized by staining with α-BTX conjugated to a fluorescent dye (33). Staining with an anti–Syne-1 antibody and tetramethylrhodamine-conjugated α-BTX revealed that Syne-1 remained localized on the NE of myonuclei in *Sun1*$^{-/-}$ mice (Fig. 1 *D* and *E*), although the expected reduction of the level of Syne-1 on synaptic nuclei could not be quantitatively determined (see below). These *Sun1*$^{-/-}$ mice exhibited a modest but significant decrease in the average number of synaptic nuclei beneath the NMJ compared with control mice of the same age (Fig. 1 *B*, *D*, and *F*); for example, in the mutant mice, 83% of the muscle fibers harbored 2 or more nuclei under the NMJ, compared with 98.2% of the muscle fibers in wild-type (WT) controls (Fig. 1*F*). Meanwhile, our quantitative analysis indicated that the distribution of nonsynaptic nuclei in *Sun1*$^{-/-}$ muscle cells was similar to that in WT controls (Fig. 1 *C* and *E*; data not shown). These results indicate that although SUN1 is not absolutely required for the NE localization of Syne-1 in skeletal muscle cells, it is required for proper synaptic nuclear anchorage. These results may suggest a role for SUN1 in maintaining a higher level of Syne-1 at synaptic nuclei, and also may indicate the involvement of another SUN protein in the recruitment of Syne-1 to the NE and myonuclear positioning.

Myonuclear Anchorage Is Not Significantly Perturbed in *Sun2* Knockout Mice. Antibody staining of SUN2 revealed that SUN2 was highly expressed and localized on the NE in muscle cells [supporting information (SI) Fig. S1*D*]. We thus generated *Sun2*$^{-/-}$ mice to investigate whether SUN2 plays a redundant role with SUN1 in myonuclear anchorage. The mutant allele was constructed by replacing the SUN domain–coding region with a *neo* cassette (Fig. S1*A*). Targeted ES clones were identified by PCR and Southern blot analysis (data not shown), and mice carrying the mutant allele were identified by genotyping PCR

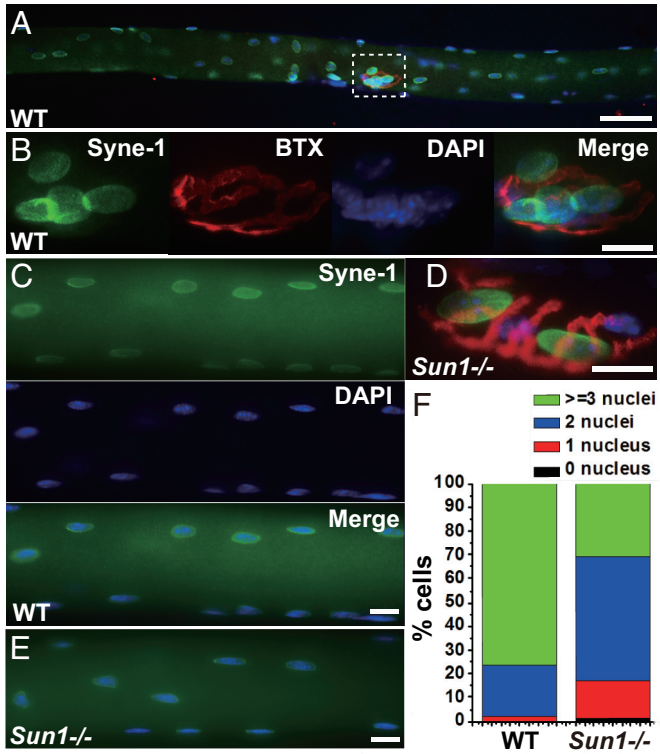

Fig. 1. The anchorage of synaptic nuclei is partially perturbed in *Sun1*$^{-/-}$ mice. (*A*) A WT muscle fiber stained with anti–Syne-1 antibody (green), α-BTX (red), and DAPI (blue). (*B*) Enlarged view of the cropped region in (*A*), showing 4 synaptic nuclei clustered beneath a representative NMJ. (*C*) Part of the same image showing an even distribution of nonsynaptic nuclei along the periphery of the muscle fibers. [Scale bars: (*A*) 40 μm; (*B* and *C*) 10 μm.] (*D* and *E*) Representative images of synaptic nuclei (*D*) and nonsynaptic nuclei (*E*) in *Sun1*$^{-/-}$ mice. Only 2 nuclei are seen under the NMJ of this *Sun1*$^{-/-}$ muscle fiber. The distribution of nonsynaptic nuclei appears to be normal. (Scale bar: 10 μm.) (*F*) Statistical data showing that the average number of synaptic nuclei is decreased in *Sun1*$^{-/-}$ muscle fibers. In *Sun1*$^{-/-}$ mice, 15.9% of the fibers contain 1 synaptic nucleus, 52.3% contain 2 synaptic nuclei, and 30.7% contain 3 or more synaptic nuclei, compared with 1.8%, 21.4%, and 76.8% of respective WT fibers (WT, $n = 56$; *Sun1*$^{-/-}$, $n = 88$; $P < .001$).

(Fig. S1*B*). The absence of the *Sun2* transcript and the SUN2 protein on the NE in skeletal muscle cells was demonstrated by RT-PCR and staining with an anti-SUN2 antibody (8), respectively (Fig. S1 *C–E*).

Unlike *Sun1*$^{-/-}$ mice, which were completely sterile (29), *Sun2*$^{-/-}$ mice were fertile and displayed no obvious abnormalities in growth and development. We previously found that SUN2 is not expressed in meiotic cells (29) (Fig. S2), suggesting that SUN2 likely does not play a critical role in meiosis. We examined both the number of synaptic nuclei and the positioning of nonsynaptic nuclei in *Sun2*$^{-/-}$ mice, and found no significant defects (Fig. S1*F*; data not shown).

The Anchorage of Synaptic Nuclei Is Severely Disrupted in *Sun1/2* Double-Knockout Mice. The overlapping expression patterns of SUN1 and SUN2 in skeletal muscles and their similar protein structures suggest that SUN1 and SUN2 may have redundant functions in myonuclear anchorage. To test this hypothesis, we combined *Sun1* and *Sun2* mutant alleles and generated *Sun1*$^{+/-}$; *Sun2*$^{-/-}$ mice, which were then intercrossed to produce *Sun1/2* double-knockout (DKO) mice. We examined more than 50 litters and found no *Sun1/2* DKO survivors. We then examined 20 litters at E18.5 and found that the number of *Sun1/2* DKO embryos was consistent with the expected Mendelian ratio (data

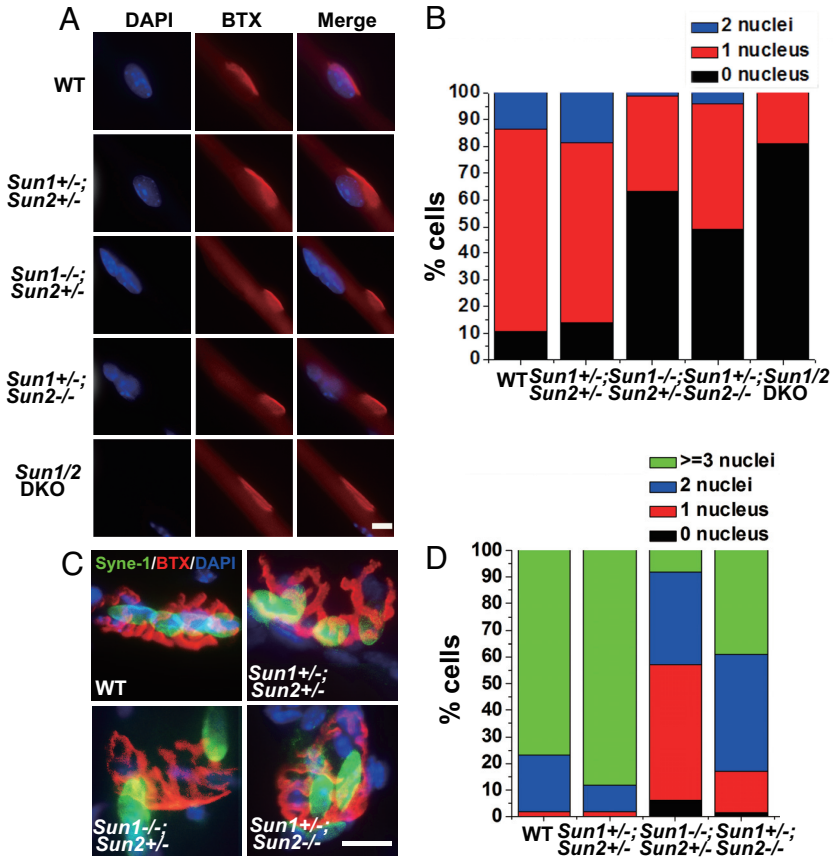

Fig. 2. SUN1 and SUN2 play partially redundant roles in anchoring synaptic nuclei. (*A*) Fluorescent images showing skeletal muscle cells of triangularis sterni in the E18.5 embryos stained with DAPI (blue) and α-BTX (red). Genotypes of the embryos are indicated. (*B*) Statistical data showing the percentage of muscle fibers harboring various numbers of synaptic nuclei in mice with different genotypes. The respective percentages of muscle fibers harboring 0, 1, or 2 synaptic nuclei are 11%, 76%, and 13% in WT mice; 14%, 67%, and 19% in *Sun1*$^{+/-}$; *Sun2*$^{+/-}$ mice; 63%, 36%, and 1% in *Sun1*$^{-/-}$; *Sun2*$^{+/-}$ mice; 49%, 47%, and 4% in *Sun1*$^{+/-}$; *Sun2*$^{-/-}$ mice; and 81%, 19%, and 0 in *Sun1/2* DKO mice. Number of muscle cells scored (*n*): WT, $n = 111$; *Sun1*$^{+/-}$; *Sun2*$^{+/-}$, $n = 122$; *Sun1*$^{-/-}$; *Sun2*$^{+/-}$, $n = 100$; *Sun1*$^{+/-}$; *Sun2*$^{-/-}$, $n = 122$; *Sun1/2* DKO, $n = 107$. (Scale bar: 5 μm.) (*C*) Representative images of synaptic myonuclei of the tibialis anterior muscle fibers in the adult mice of various genotypes stained with the anti–Syne-1 antibody (green), α-BTX (red), and DAPI (blue). (Scale bar: 10 μm.) (*D*) Statistical data showing the percentages of muscle fibers harboring various numbers of synaptic nuclei in mice with different genotypes. The respective percentages of muscle fibers harboring 0, 1, 2, or 3 or more synaptic nuclei are 0%, 1.8%, 21.4%, and 76.8% in WT mice (the same data as in Fig. 1*F*); 0%, 1.7%, 10.2%, and 88.1% in *Sun1*$^{+/-}$; *Sun2*$^{+/-}$ mice; 6.1%, 51%, 34.7%, and 8.2% in *Sun1*$^{-/-}$; *Sun2*$^{+/-}$ mice; and 1.6%, 15.6%, 43.8%, and 39% in *Sun1*$^{+/-}$; *Sun2*$^{-/-}$ mice. Number of muscle cells scored: WT, $n = 56$; *Sun1*$^{+/-}$; *Sun2*$^{+/-}$, $n = 59$; *Sun1*$^{-/-}$; *Sun2*$^{+/-}$, $n = 49$; *Sun1*$^{+/-}$; *Sun2*$^{-/-}$, $n = 64$. These data indicate that both SUN1 and SUN2 contribute significantly to the anchorage of synaptic nuclei.

not shown). Further analysis found that the *Sun1/2* DKO pups reached full term but died soon after birth (Fig. S3).

To assess the anchorage of myonuclei in *Sun1/2* DKO embryos, we dissected out the triangularis sterni from the E18.5 embryos and stained them with DAPI and α-BTX. More than 100 cells with each genotype were examined. One or 2 nuclei were found to lie beneath each NMJ in the muscle fibers of WT and *Sun1*$^{+/-}$; *Sun2*$^{+/-}$ mice (Fig. 2 *A* and *B*). In contrast, 63% of the muscle cells in *Sun1*$^{-/-}$; *Sun2*$^{+/-}$ mice and 49% of those in *Sun1*$^{+/-}$; *Sun2*$^{-/-}$ mice harbored no nuclei beneath the NMJ (Fig. 2 *A* and *B*; $P < .001$). The anchorage defect was exacerbated in the muscle fibers of *Sun1/2* DKO mice, where only 19% of the muscle cells contained 1 nucleus beneath the NMJ (Fig. 2 *A* and *B*; $P < .001$). Next, we examined the anchorage of synaptic nuclei in adult mice, and found significant defects in *Sun1*$^{-/-}$; *Sun2*$^{+/-}$ mice and *Sun1*$^{+/-}$; *Sun2*$^{-/-}$ mice compared with WT and *Sun1*$^{+/-}$; *Sun2*$^{+/-}$ mice (Figs. 1*F* and 2 *C* and *D*; $P < .001$). These gene dosage–dependent effects indicate that SUN1 and SUN2 have partially redundant functions on synaptic nuclear anchorage.

The Organization of Nonsynaptic Nuclei Is Disrupted in *Sun1/2* DKO Mice. Because the *Sun1/2* DKO mice died shortly after birth, when the muscle fibers were not yet completely developed, examining the distribution of the nonsynaptic nuclei was not feasible. We examined adult mice with the genotype of WT, *Sun1*$^{+/-}$; *Sun2*$^{+/-}$, *Sun1*$^{-/-}$; *Sun2*$^{+/-}$, and *Sun1*$^{+/-}$; *Sun2*$^{-/-}$ and found a normal distribution of nonsynaptic nuclei in these mice (Fig. 3 *A–D* and *G*). Speculating that the lethality of *Sun1/2* DKO mice might be due to unknown defects in the central nervous system, we generated transgenic mice that expressed the *Sun1* gene driven by the neuron-specific enolase (NSE) promoter. The NSE promoter, derived from the rat NSE gene, has been shown to drive transgene expression in a broad range of neurons in mice (34). The *Sun1*$^{-/-}$; *Sun2*$^{-/-}$; *NES*::*Sun1* (RDKO) mice were alive at birth, and some mice with this genotype survived to adulthood (Fig. 4). The nuclear positioning defects in skeletal muscles were not rescued in these mice (not shown), consistent with the lack of transgene expression in muscle cells (Fig. 4). This gave us the opportunity to quantitatively examine the positioning of nonsynaptic nuclei in adult mice in the absence of any of the WT *Sun1/2* alleles in skeletal muscle cells. We found that on average, more than 3 nuclear clusters were present in each muscle cell of the RDKO mice (Fig. 3 *F* and *G*); a nuclear cluster is defined as 3 or more nuclei grouped together, with the distance between adjacent nuclei less than their diameter. This clustering

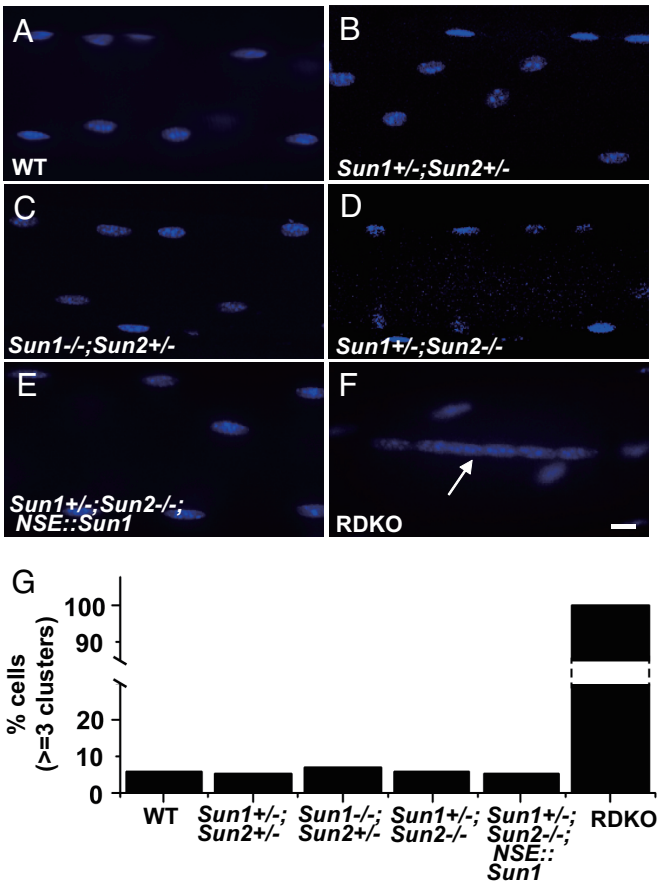

Fig. 3. The organization of nonsynaptic nuclei is disrupted in $^{-/-;-/-}$RDKO mice. (*A–F*) The distribution of nonsynaptic nuclei in skeletal muscle fibers of adult mice stained with DAPI (blue). The arrow indicates a typical cluster of 6 nonsynaptic nuclei. (*G*) Statistical data showing the formation of myonuclear clusters in muscle fibers of $^{-/-;-/-}$RDKO mice. Number of muscle fibers scored: WT, $n = 101$; *Sun1*$^{+/-}$; *Sun2*$^{+/-}$, $n = 104$; *Sun1*$^{-/-}$; *Sun2*$^{+/-}$, $n = 105$; *Sun1*$^{+/-}$; *Sun2*$^{-/-}$, $n = 109$; *Sun1*$^{+/-}$; *Sun2*$^{-/-}$; *NSE*::*Sun1*: $n = 102$; RDKO, $n = 50$. (Scale bar: 10 μm.)

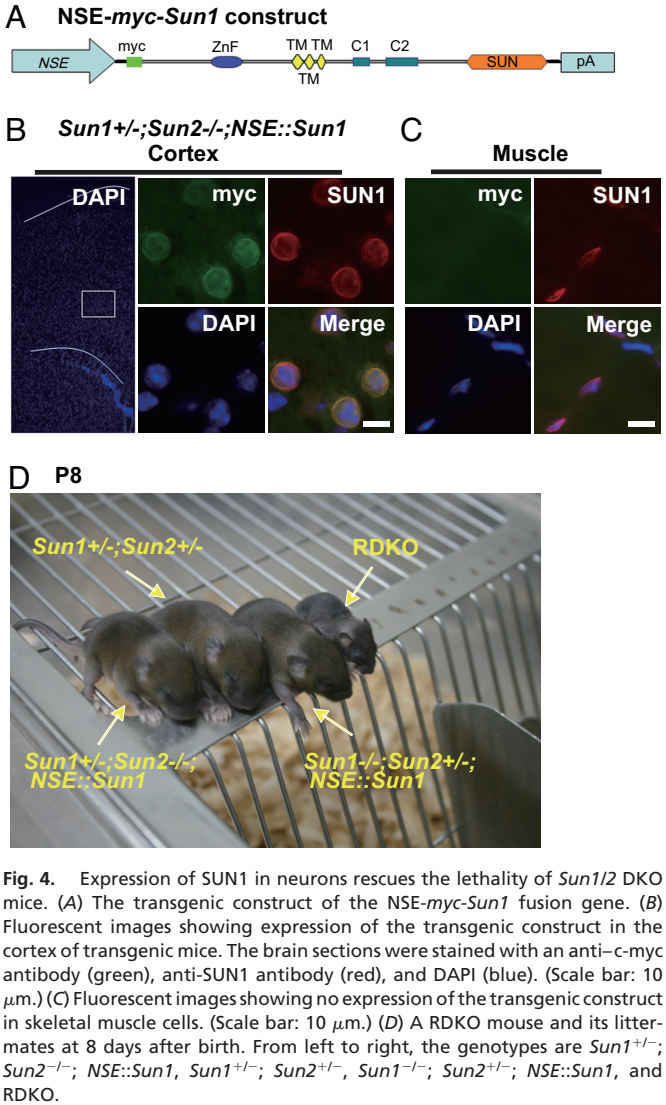

Fig. 4. Expression of SUN1 in neurons rescues the lethality of *Sun1/2* DKO mice. (*A*) The transgenic construct of the NSE-*myc-Sun1* fusion gene. (*B*) Fluorescent images showing expression of the transgenic construct in the cortex of transgenic mice. The brain sections were stained with an anti–c-myc antibody (green), anti-SUN1 antibody (red), and DAPI (blue). (Scale bar: 10 μm.) (*C*) Fluorescent images showing no expression of the transgenic construct in skeletal muscle cells. (Scale bar: 10 μm.) (*D*) A RDKO mouse and its littermates at 8 days after birth. From left to right, the genotypes are *Sun1*$^{+/-}$; *Sun2*$^{-/-}$; *NSE*::*Sun1*, *Sun1*$^{+/-}$; *Sun2*$^{+/-}$, *Sun1*$^{-/-}$; *Sun2*$^{+/-}$; *NSE*::*Sun1*, and RDKO.

phenotype was not caused by a novel effect of the transgene itself, because the distribution of nonsynaptic nuclei in the control *Sun1*$^{+/-}$; *Sun2*$^{-/-}$;*NSE*::*Sun1* mice was normal (Fig. 3 *E* and *G*).

The NE Localization of Syne-1, But Not Lamin A/C, Is Disrupted in *Sun1/2* DKO Mice. Given the similar myonuclear anchorage defects of *Sun1/2* DKO mice and *Syne-1* KASH deletion (*Syne-1*$^{-/-}$) mice, as well as the relationship between KASH proteins and SUN proteins (13, 27), we examined the expression of Syne-1 in skeletal muscle cells of mice harboring the *Sun1* and *Sun2* mutant alleles. The NE localization of Syne-1 appeared to be normal in *Sun1* and *Sun2* double-heterozygous mice (Fig. 5 *D–F*); however, the NE-localized Syne-1 signal in skeletal muscle cells appeared to be reduced in *Sun1*$^{+/-}$; *Sun2*$^{-/-}$ and *Sun1*$^{-/-}$; *Sun2*$^{+/-}$ mice and was completely absent in *Sun1/2* DKO mice (Fig. 5 *G–O*). These results indicate that SUN1 and SUN2 play a critical but redundant role in recruiting Syne-1 to the NE of skeletal muscle cells.

We also examined the NE localization of SUN1 and SUN2 in the muscle fibers of Syne-1/2 double KASH deletion (*Syne-1*$^{-/-}$; *Syne-2*$^{-/-}$) mice and found no abnormalities (Fig. S4). This finding indicates that these 2 KASH proteins are not required for NE localization of the 2 SUN proteins.

Previous studies have shown that the SUN proteins can interact with nuclear lamina proteins and that the loss of lamin A/C disrupts myonuclear anchorage in mice (25, 28, 35). We examined the localization of Lamin A/C in skeletal muscle cells of *Sun1/2* DKO mice. Compared with heterozygotes, the NE localization of Lamin A/C was not disturbed in *Sun1/2* DKO mice (Fig. 5 *P–T*). This suggests that SUN1 and SUN2 are not essential for NE localization of Lamin A/C, in agreement with findings from studies in other organisms and tissue culture cells (15, 24, 25, 28, 36).

Discussion

Previous studies on KASH and SUN proteins have provided evidence of their interaction at the NE in several cellular processes (10, 12–15, 17, 20). Syne-1 has been shown to be essential for proper myonuclear anchorage. Our findings provide conclusive evidence to support the idea that SUN1 and SUN2 function redundantly in the anchorage of myonuclei, including synaptic and nonsynaptic nuclei. They also show that SUN1 and SUN2 act redundantly to recruit Syne-1 to the NE.

SUN1 and SUN2 have been shown to be inner NE proteins, with their conserved C terminus SUN domain at the lumen of the NE and the N terminus in the nucleus (26, 28). They can form homodimers and heterodimers in tissue culture cells (28, 37). Our data indicate that both proteins are highly expressed and

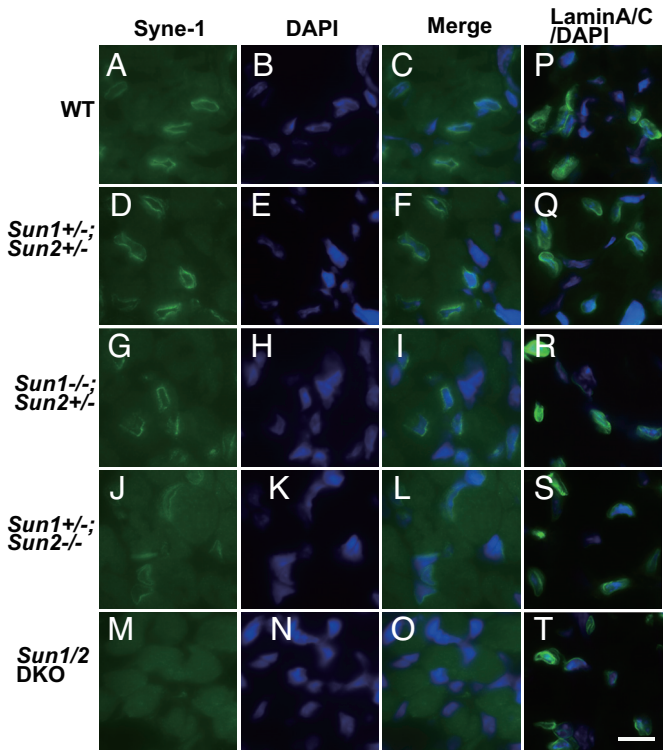

Fig. 5. The NE localization of Syne-1 but not Lamin A/C is disrupted in *Sun1/2* DKO mice. (*A–O*) Representative images of intercostal muscle sections of the E18.5 embryos stained with the anti–Syne-1 antibody (green) and DAPI (blue). Genotypes of the embryos are indicated. Syne-1 displays the NE localization pattern in muscle cells in WT (*A–C*), *Sun1*$^{+/-}$; *Sun2*$^{+/-}$ (*D–F*), *Sun1*$^{-/-}$; *Sun2*$^{+/-}$ (*G–I*), and *Sun1*$^{+/-}$; *Sun2*$^{-/-}$ (*J–L*) mice, but not in the *Sun1/2* DKO cells (*M–O*). (*P–T*) Representative merged images of intercostal muscle sections of the E18.5 embryos stained with the anti-Lamin A/C antibody (green) and DAPI (blue). Lamin A/C displays NE localization in muscle cells of all genotypes. (Scale bar: 10 μm.)

localized on the NE of muscle cells. These features underlie the redundant roles of SUN1 and SUN2 in skeletal muscle cells.

Investigation of the physiological function of myonuclear anchorage has been an interesting and difficult problem. Previously, it was speculated that synaptic nuclei have specialized roles in maintaining the high expression levels of postsynaptic components and thus the proper formation of the NMJ (1, 38); however, our previous study of the Syne-1/Nesprin-1 protein indicated that neither the formation of the NMJ nor the expression of several synaptic components (synaptophysin, AchR, rapsyn, MuSK, and utrophin) was obviously disrupted (7, 8). In the present study, we also examined the expression of rapsyn, synaptophysin, and utrophin in E18.5 skeletal muscles and found no obvious decrease in these protein levels at the NMJ in the *Sun1/2* DKO mice (Fig. S5). Moreover, the lack of prominent developmental defects in the Syne-1/Nesprin-1 KASH deletion mice also suggests that myonuclear anchorage is not essential for mouse development. We did find disturbed formation of proper muscle–nerve connections due to the altered positions of the NMJ within individual mutant myofibers, however (ref. 8 and data not shown). The effects of this abnormal myonuclear positioning on animal development and behavior, as well as other important physiological effects of disrupted myonuclear anchorage, remain to be determined. It is important to note that Syne-1/Nesprin-1 does play essential roles (although redundantly with Syne-2/Nesprin-2) in developmental aspects other than myonuclear anchorage, as clearly indicated by the dramatic lethal phenotype of Syne-1 and Syne-2 double KASH deletion mutants (8).

KASH–SUN protein complexes also have been implicated in roles related to muscular dystrophies (9, 39). The degenerative phenotype may be related to the defects in myonuclear anchorage, including nonsynaptic anchorage. It is interesting that in a recent report, independently generated mice with deletions in the C-terminal KASH domain exhibited more severe developmental and behavior defects than the mice that we generated previously (refs. 8 and 9; L.K. and X.Z., unpublished data). Such a difference might be linked to the different genetic backgrounds of the mice or to mutations induced in ES cells during the targeting process (40), possibly suggesting that mice become highly dependent on the Syne-1/Nesprin-1 function when certain other cellular functions are compromised. Alternatively, the variation in phenotypes could be due to the small difference in the deletion alleles generated in the 2 Syne-1/Nesprin-1 strains. For example, 1 of the alleles might disrupt a KASH domain–independent function. In humans, mutations in Syne-1/Nesprin-1 have been associated with a certain type of recessive cerebellar ataxia (41). Although the precise mechanism of this disease is not clear, it is unlikely to be caused primarily by the defect in nuclear anchorage in muscle cells, based on our analysis of the Syne-1/Nesprin-1 KASH deletion mice (data not shown).

Our observations that the neonatal lethality of *Sun1/2* DKO mice was rescued by expressing the *Sun1* gene driven by a neuron-specific promoter (RDKO mice) indicates that SUN1 and SUN2 likely play important but redundant roles in the development of the central nervous system. However, the RDKO mice had a smaller body size (Fig. 4) and exhibited defects on a neuronal behavior assay (e.g., feet clasping; data not shown), suggesting that the *NSE*::*Sun1* transgene may not have rescued all neuronal defects in the *Sun1/2* DKO mice. The roles of SUN1 and SUN2 in brain development are currently under investigation using *Sun1/2* DKO as well as conditional KO mice.

Materials and Methods

Generation of *Sun2* Knockout Mice and *Sun1/2* DKO Mice. To construct the *Sun2* gene targeting vector, the 11.5-kb BamH I DNA fragment containing exons 5–10 of *Sun2* and the 1.4-kb EcoR I fragment containing part of exon 17 were obtained from a BAC clone of the Sv129 strain (no. 49709; Invitrogen). These 2 fragments were cloned into a pPNT vector, flanking the pgk-*Neo* cassette as the targeting arms. The resulting targeting vector was linearized and electroporated into W4/129S6 ES cells (Taconic Transgenics). After 2 rounds of selection, the ES clones were screened for successful targeting by PCR analysis using primers prDX016 and prDX081. The positive ones were further identified by Southern blot analysis using a 778-bp genomic fragment as the probe (PCR-amplified with primers prDX086 and prDX087). The targeted ES clones were injected into C57BL/6J blastocysts. The genotype of the targeted *Sun2* allele in mice was determined by PCR analysis using primers prDX016, prDX081, and prDX115. *Sun1/2* DKO mice were generated by intercrossing *Sun1*$^{-/-}$ and *Sun2*$^{-/-}$ mice. The primer sequences used are listed in SI *Materials and Methods*.

Mouse breeding and experimental manipulations were carried out following the general guidelines published by the Association for Assessment and Accreditation of Laboratory Animal Care. All animal-related procedures were reviewed and approved by the Institute of Developmental Biology and Molecular Medicine Institutional Animal Care and Use Committee.

Immunofluorescent Staining and Microscopic Analysis. Immunofluorescent staining of frozen sections was carried out following standard protocols (42). Whole-mount staining of skeletal muscle cells was carried out as described previously (7, 8). To isolate individual myofibers from E18.5 embryos, the thoraxes were fixed and triangularis sterni were extracted. After staining with tetramethylrhodamine-conjugated α-BTX (Molecular Probes) and DAPI, individual fibers were teased out and mounted in the mounting medium (Sigma). At the E18.5 stage, the motor neuron has not yet invaginated into the muscle fiber. When triangularis stermi were dissected out to obtain the single muscle fiber, a clean separation between the fiber and surrounding cell/nuclei was usually achieved. Thus, the distraction of nonmuscle nuclei in these experiments was minimal and did not present a significant problem for quantitative

scoring of the mutant phenotype. For scoring the nonsynaptic nuclei clusters in adult mice, we chose only fibers with minimal contamination of nonmuscle cells. The reliability of the phenotype scoring is supported by the fact that we did not detect a single prominent nuclear cluster in the WT fibers that we examined, and that the clustering phenotype was obvious and highly penetrant in the RDKO mice. The antibodies used in this study were mouse anti-Lamin A/C (Chemicon), goat anti-mouse IgG-FITC (Chemicon), goat anti-rabbit IgG-FITC (Sigma), mouse anti-rapsyn (Sigma), rabbit anti-synaptophysin (Zymed), and mouse anti-utrophin (Novocastra). The production of antibodies against SUN1, SUN2, and Syne-1 has been described previously (8, 29).

Photographs were taken with a Leica DMRXA2 system. Images were manipulated with Leica FW4000, Adobe Photoshop software, and Adobe Illustrator software.

ACKNOWLEDGMENTS. We thank Xiaohui Wu, Yanling Yang, and Yanfeng Tan for their help in producing the chimeras and transgenic mice and Beibei Ying, Xiaohui Wu, Kejing Deng, Lin Sun, Wufan Tao, Aileen Sewell, and other members of the IDM for their assistance and discussions. This work was supported by grants from the National Natural Science Foundation of China, the Chinese Ministry of Science and Technology (973 Program) and Ministry of Education (211 Program), and the Shanghai Municipal Government.

1. Sanes JR, Lichtman JW (2001) Induction, assembly, maturation and maintenance of a postsynaptic apparatus. *Nat Rev Neurosci* 2:791–805.
2. Bruusgaard JC, Liestol K, Ekmark M, Kollstad K, Gundersen K (2003) Number and spatial distribution of nuclei in the muscle fibres of normal mice studied in vivo. *J Physiol* 551(Pt 2):467–478.
3. Starr DA, Fischer JA (2005) KASH 'n karry: The KASH domain family of cargo-specific cytoskeletal adaptor proteins. *Bioessays* 27:1136–1146.
4. Tzur YB, Wilson KL, Gruenbaum Y (2006) SUN-domain proteins: ''Velcro'' that links the nucleoskeleton to the cytoskeleton. *Nat Rev Mol Cell Biol* 7:782–788.
5. Wilhelmsen K, Ketema M, Truong H, Sonnenberg A (2006) KASH-domain proteins in nuclear migration, anchorage and other processes. *J Cell Sci* 119(Pt 24):5021–5029.
6. Starr DA (2009) A nuclear-envelope bridge positions nuclei and moves chromosomes. *J Cell Sci* 122(Pt 5):577–586.
7. Grady RM, Starr DA, Ackerman GL, Sanes JR, Han M (2005) Syne proteins anchor muscle nuclei at the neuromuscular junction. *Proc Natl Acad Sci USA* 102:4359–4364.
8. Zhang X, et al. (2007) Syne-1 and Syne-2 play crucial roles in myonuclear anchorage and motor neuron innervation. *Development* 134:901–908.
9. Puckelwartz MJ, et al. (2009) Disruption of nesprin-1 produces an Emery Dreifuss muscular dystrophy–like phenotype in mice. *Hum Mol Genet* 18(4):607–620.
10. Malone CJ, Fixsen WD, Horvitz HR, Han M (1999) UNC-84 localizes to the nuclear envelope and is required for nuclear migration and anchoring during *C. elegans* development. *Development* 126:3171–3181.
11. Worman HJ, Gundersen GG (2006) Here come the SUNs: A nucleocytoskeletal missing link. *Trends Cell Biol* 16(2):67–69.
12. Starr DA, et al. (2001) UNC-83 encodes a novel component of the nuclear envelope and is essential for proper nuclear migration. *Development* 128:5039–5050.
13. Starr DA, Han M (2002) Role of ANC-1 in tethering nuclei to the actin cytoskeleton. *Science* 298:406–409.
14. McGee MD, Rillo R, Anderson AS, Starr DA (2006) UNC-83 is a KASH protein required for nuclear migration and is recruited to the outer nuclear membrane by a physical interaction with the SUN protein UNC-84. *Mol Biol Cell* 17:1790–1801.
15. Kracklauer MP, Banks SM, Xie X, Wu Y, Fischer JA (2007) *Drosophila klaroid* encodes a SUN domain protein required for Klarsicht localization to the nuclear envelope and nuclear migration in the eye. *Fly (Austin)* 1:75–85.
16. Hagan I, Yanagida M (1995) The product of the spindle formation gene *sad1+* associates with the fission yeast spindle pole body and is essential for viability. *J Cell Biol* 129:1033–1047.
17. Malone CJ, et al. (2003) The *C. elegans* hook protein, ZYG-12, mediates the essential attachment between the centrosome and nucleus. *Cell* 115:825–836.
18. Fridkin A, et al. (2004) Matefin, a *Caenorhabditis elegans* germ line–specific SUN-domain nuclear membrane protein, is essential for early embryonic and germ cell development. *Proc Natl Acad Sci USA* 101:6987–6992.
19. Chikashige Y, et al. (2006) Meiotic proteins bqt1 and bqt2 tether telomeres to form the bouquet arrangement of chromosomes. *Cell* 125:59–69.
20. Jaspersen SL, et al. (2006) The Sad1–UNC-84 homology domain in Mps3 interacts with Mps2 to connect the spindle pole body with the nuclear envelope. *J Cell Biol* 174:665–675.
21. Penkner A, et al. (2007) The nuclear envelope protein Matefin/SUN-1 is required for homologous pairing in *C. elegans* meiosis. *Dev Cell* 12:873–885.
22. King MC, Drivas TG, Blobel G (2008) A network of nuclear envelope membrane proteins linking centromeres to microtubules. *Cell* 134:427–438.
23. Shao X, Tarnasky HA, Lee JP, Oko R, van der Hoorn FA (1999) Spag4, a novel sperm protein, binds outer dense-fiber protein Odf1 and localizes to microtubules of manchette and axoneme. *Dev Biol* 211:109–123.
24. Crisp M, et al. (2006) Coupling of the nucleus and cytoplasm: Role of the LINC complex. *J Cell Biol* 172:41–53.
25. Hasan S, et al. (2006) Nuclear envelope localization of human UNC84A does not require nuclear lamins. *FEBS Lett* 580:1263–1268.
26. Hodzic DM, Yeater DB, Bengtsson L, Otto H, Stahl PD (2004) Sun2 is a novel mammalian inner nuclear membrane protein. *J Biol Chem* 279:25805–25812.
27. Padmakumar VC, et al. (2005) The inner nuclear membrane protein Sun1 mediates the anchorage of Nesprin-2 to the nuclear envelope. *J Cell Sci* 118(Pt 15):3419–3430.
28. Haque F, et al. (2006) SUN1 interacts with nuclear lamin A and cytoplasmic nesprins to provide a physical connection between the nuclear lamina and the cytoskeleton. *Mol Cell Biol* 26:3738–3751.
29. Ding X, et al. (2007) SUN1 is required for telomere attachment to nuclear envelope and gametogenesis in mice. *Dev Cell* 12:863–872.
30. Chi YH, et al. (2009) Requirement for Sun1 in the expression of meiotic reproductive genes and piRNA. *Development* 136:965–973.
31. Luke Y, et al. (2008) Nesprin-2 giant (NUANCE) maintains nuclear envelope architecture and composition in skin. *J Cell Sci* 121(Pt 11):1887–1898.
32. Apel ED, Lewis RM, Grady RM, Sanes JR (2000) Syne-1, a dystrophin- and Klarsicht-related protein associated with synaptic nuclei at the neuromuscular junction. *J Biol Chem* 275:31986–31995.
33. Wigston DJ (1990) Repeated in vivo visualization of neuromuscular junctions in adult mouse lateral gastrocnemius. *J Neurosci* 10:1753–1761.
34. Forss-Petter S, et al. (1990) Transgenic mice expressing beta-galactosidase in mature neurons under neuron-specific enolase promoter control. *Neuron* 5:187–197.
35. Mejat A, et al. (2009) Lamin A/C–mediated neuromuscular junction defects in Emery-Dreifuss muscular dystrophy. *J Cell Biol* 184:31–44.
36. Lee KK, et al. (2002) Lamin-dependent localization of UNC-84, a protein required for nuclear migration in *Caenorhabditis elegans*. *Mol Biol Cell* 13:892–901.
37. Wang Q, Du X, Cai Z, Greene MI (2006) Characterization of the structures involved in localization of the SUN proteins to the nuclear envelope and the centrosome. *DNA Cell Biol* 25:554–562.
38. Schaeffer L, de Kerchove d'Exaerde A, Changeux JP (2001) Targeting transcription to the neuromuscular synapse. *Neuron* 31:15–22.
39. Zhang Q, et al. (2007) Nesprin-1 and -2 are involved in the pathogenesis of Emery Dreifuss muscular dystrophy and are critical for nuclear envelope integrity. *Hum Mol Genet* 16:2816–2833.
40. Liang Q, Conte N, Skarnes WC, Bradley A (2008) Extensive genomic copy number variation in embryonic stem cells. *Proc Natl Acad Sci USA* 105:17453–17456.
41. Dupre N, Bouchard JP, Gros-Louis F, Rouleau GA (2007) Mutations in SYNE-1 lead to a newly discovered form of autosomal recessive cerebellar ataxia. *Med Sci (Paris)* 23:261–262 (in French).
42. Harlow DL, ed (1999) *Using Antibodies*: *A Lab Manual* (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY).